METHOD OF DIAGNOSING AND TREATING BALDNESS USING HUMAN AND MOUSE RHOR GENE AND CODED PRODUCT THEREOF

FIELD OF INVENTION

This invention relates to biological and medical fields. In particular, it relates to a novel polynucleotide encoding baldness-related protein and the polypeptide encoded by the polynucleotide. The invention also relates to the uses and preparation of these polynucleotides and polypeptides.

TECHNICAL BACKGROUND

The human primary baldness and loss of hair are quite common in the males, but are rare in females. Although baldness does not influence the survival of human living, it may severely influence the quality of individual life, especially for female patients suffering from this inherent genetic disease. Unfortunately, up to now, there is no way to completely cure baldness. Furthermore, it is very difficult to get the family suffering primary baldness.

Up to now, no baldness related gene has been isolated or discovered. Therefore, there is an urgent need to develop new baldness-related proteins.

SUMMARY OF INVENTION

One purpose of the invention is to provide a baldness-related protein, which was named Rhor protein, and its fragments, analogs and derivatives.

Another purpose of the invention is to provide a polynucleotide encoding said polypeptides.

Still another purpose of the invention is to provide a method for preparing said polypeptides and the uses of said polypeptides and their encoding sequences.

In the 1st aspect, the invention provides an isolated Rhor polypeptide, which comprises a polypeptide having the amino acid sequence of SEQ ID NO: 2, its conservative variants, its active fragments, and its active derivatives. Preferably, said polypeptide is a polypeptide having the amino acid sequence of SEQ ID NO: 2.

In the 2nd aspect, the invention provides an isolated polynucleotide, which comprises a nucleotide sequence sharing at least 85% homology to the following nucleotide sequence: (a) the nucleotide sequence encoding the above Rhor polypeptide; (b) the polynucleotide complementary to nucleotide sequence of (a). Preferably, said nucleotide sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 2. More preferably, said polynucleotide comprises nucleotides 1-2484 of SEQ ID NO: 1.

In the 3rd aspect, the invention provides a vector comprising the above polynucleotide, and a host cell transformed with said vector or a host cell transformed with said polynucleotide.

In the 4th aspect, the invention provides a method for producing a polypeptide having the

activity of Rhor protein, which comprises:

(a) culturing the above transformed host cell under the conditions suitable for the expression of protein;

(b) isolating the polypeptides having the activity of Rhor protein from the culture.

In the 5th aspect, the invention provides an antibody specifically bound to Rhor protein. Also provided are nucleic acid molecules for detection, which comprises consecutive 20-2484 nucleotides of the above polynucleotide.

In the 6th aspect, it provides compounds that simulate, promote and antagonize Rhor activity, or inhibit Rhor expression and methods for screening and preparing these compounds. Preferably, the compounds are antisense sequences of Rhor encoding sequence or fragments thereof.

In the 7th aspect, it provides a method for detecting Rhor protein in a sample, comprising: contacting the sample with the antibody specifically against Rhor protein, observing the formation of antibody complex which indicates the presence of Rhor protein in the sample.

In the 8th aspect, it provides a method for determining the diseases related to Rhor abnormal expression (e.g., baldness) or the susceptibility thereof, which comprises detecting the mutation of Rhor encoding sequence. Preferably, said mutation is deletion of nucleotides 351-580 of SEQ ID NO: 1. Further, the invention provides a kit for detecting baldness comprising the primers which specifically amplify the Rhor gene or transcript. Preferably, the kit further comprises a probe that binds to the site of mutation.

In the 9th aspect, it provides the uses of Rhor and its encoding sequence, e.g., in screening Rhor agonists and antagonist, and peptide fingerprinting. The Rhor encoding sequence and its fragment can be used as primers in PCR, or probes in hybridization and microarray.

In the 10th aspect, it provides a pharmaceutical composition comprising a safe and efficient amount of Rhor protein, or its agonist or antagonist and pharmaceutically acceptable carrier. This pharmaceutical composition can be used to treat diseases, e.g., baldness.

The other aspects of the invention will be apparent to the skilled in the art in light of the technical disclosure of the invention.

DESCRIPTION OF DRAWINGS

Fig. 1 shows the PCR amplification results of Rhor gene in different mice. Lane 1: mice without fair; Lane 2: mice with litter fare; Lane 3 and 4: normal mice.

DETAILED DESCRIPTION OF INVENTION

After comprehensive and extensive study, the inventors have first isolated and identified a new gene Rhor, which is close related to baldness. Rhor gene encodes a protein similar to EGF receptor. The mutation of Rhor as well the decrease or loss of Rhor function directly result in baldness. On basis of said discovery, the inventors completed this invention.

As used herein, the term "Rhor protein", "Rhor polypeptide" or "baldness-related protein Rhor" are exchangeable, referring to a protein or polypeptide comprising the amino acid sequence of natural baldness-related peptide Rhor (SEQ ID NO: 2). The term includes Rhor protein with or without the starting Met residue, Rhor protein with or without signal peptide.

As used herein, the term "isolated" refers to a substance which has been isolated from the original environment. For naturally occurring substance, the original environment is the natural environment. E.g., the polynucleotide and polypeptide in a naturally occurring state in the viable cells are not isolated or purified. However, if the same polynucleotide and polypeptide have been isolated from other components naturally accompanying them, they are isolated or purified.

As used herein, the terms "isolated Rhor protein or polypeptide" mean that Rhor polypeptide does not essentially contain other proteins, lipids, carbohydrate or any other substances associated therewith in nature. The artisans can purify Rhor protein by standard protein purification techniques. Essentially purified polypeptide forms a single main band on a non-reductive PAGE gel.

The polypeptide of invention may be a recombinant, natural, or synthetic polypeptide, preferably a recombinant polypeptide. The polypeptide of invention may be a purified natural product or a chemically synthetic product. Alternatively, it may be produced from prokaryotic or eukaryotic hosts, such as bacteria, yeast, higher plant, insect, and mammalian cells, using recombinant techniques. According to the host used in the recombinant production, the polypeptide may be glycosylated or non-glycosylated.

The invention further comprises the fragments, derivatives and analogues of Rhor. As used in the invention, the terms " fragment ", " derivative " and " analogue " mean the polypeptide that essentially retains the same biological functions or activity of natural Rhor protein. The fragment, derivative or analogue of the polypeptide may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), or (ii) one in which one or more of the amino acid residues include a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to mature polypeptide, such as a leader or secretary sequence or a sequence used for purifying polypeptide or proprotein, e.g., a fusion protein formed with IgC fragment. Such fragments, derivatives and analogs are known to the artisans based on the teachings herein.

In the present invention, the term "Rhor polypeptide" means a polypeptide having the activity of Rhor protein comprising the amino acid sequence of SEQ ID NO: 2. The term also comprises the variants which have the same function of Rhor. These variants include, but are not limited to, deletions, insertions and/or substitutions of several amino acids (typically 1-50, preferably 1-30, more preferably 1-20, most preferably 1-10), and addition of one or more amino acids (typically less than 20, preferably less than 10, more preferably less than 5) at C-terminal and/or N-terminal. E.g., the protein functions are usually unchanged when an amino residue is substituted by a similar or

analogous one. Further, the addition of one or several amino acids at C-terminal and/or N-terminal usually does not change the protein function. The term also includes the active fragments and derivatives of Rhor protein.

The variants of polypeptide include homologous sequences, conservative mutants, allelic variants, natural mutants, induced mutants, proteins encoded by DNA which hybridizes to Rhor DNA under high or low stringency conditions as well as the polypeptides retrieved by antisera raised against Rhor polypeptide. The present invention also provides other polypeptides, e.g., fusion proteins, which include the Rhor polypeptide or fragments thereof. Besides substantially full-length polypeptide, the soluble fragments of Rhor polypeptide are also included. Generally, these fragments comprise at least 10, typically at least 30, preferably at least 50, more preferably at least 80, and most preferably at least 100 consecutive amino acids of Rhor polypeptide.

The invention also provides the analogues of Rhor polypeptide. Analogues can differ from naturally occurring Rhor polypeptide by amino acid sequence differences or by modifications which do not affect the sequence, or by both. These polypeptides include genetic variants, both natural and induced. Induced variants can be made by various techniques, e.g., by random mutagenesis using irradiation or exposure to mutagens, or by site-directed mutagenesis or other known molecular biologic techniques. Also included are analogues which include residues other than those naturally occurring L-amino acids (e.g., D-amino acids) or non-naturally occurring or synthetic amino acids (e.g., beta- or gamma-amino acids). It is understood that the polypeptides of the invention are not limited to the representative polypeptides listed hereinabove.

Modifications (which do not normally alter primary sequence) include in vivo or *in vitro* chemical derivation of polypeptides, e.g., acelylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in the further processing steps, e.g., by exposing the polypeptide to glycosylation enzymes (e.g., mammalian glycosylating or deglycosylating enzymes). Also included are sequences having phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, phosphothronine, as well as sequences modified to improve the resistance to proteolytic degradation or to optimize solubility properties.

In the invention, "Rhor conservative mutant" means a polypeptide formed by substituting at most 10, preferably at most 8, more preferably 5, and most preferably at most 3 amino acids with the amino acids having substantially the same or similar property, as compared with the amino acid sequence of SEQ ID NO: 2. Preferably, these conservative mutants are formed by the substitution according to Table 1.

Table 1

Initial residue	Representative substitution	Preferred substitution
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Lys; Arg	Gln

Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro; Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe	Leu
Leu (L)	Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Leu; Val; Ile; Ala; Tyr	Leu
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala	Leu

The polynucleotide of invention may be in the forms of DNA and RNA. DNA includes cDNA, genomic DNA, and synthetic DNA, etc., in single strand or double strand form. A single strand DNA may be an encoding strand or non-encoding strand. The coding sequence for mature polypeptide may be identical to the coding sequence shown in SEQ ID NO:1, or is a degenerate sequence. As used herein, the term "degenerate sequence" means an sequence which encodes a protein comprising the sequence of SEQ ID NO: 2 and which has a nucleotide sequence different from the coding region in SEQ ID NO:1.

The sequences encoding the mature polypeptide include those encoding only the mature polypeptide, those encoding mature polypeptide plus various additional encoding sequence, the encoding sequence for mature polypeptide plus the non-encoding sequence and optional additional encoding sequence.

The term "polynucleotide encoding the polypeptide " includes the polynucleotide encoding said polypeptide and the polynucleotide comprising additional and/or non-encoding sequence.

The invention further relates to the variants of polynucleotides which encode a polypeptide having the same amino acid sequence, or its fragment, analogue and derivative. The variant of the polynucleotide may be a naturally occurring allelic variant or a non-naturally occurring variant. Such nucleotide variants include substitution, deletion, and insertion variants. As known in the art, the allelic variant is a substitution form of polynucleotide, which may be a substitution, deletion, and insertion of one or more nucleotides without substantially changing the functions of the encoded

polypeptide.

The invention further relates to polynucleotides, which hybridize to the hereinabove-described sequences, if there is at least 50%, preferably at least 70%, and more preferably at least 80% between the sequences. The invention particularly relates to polynucleotides, which hybridize under stringent conditions to the polynucleotides of the invention. As herein used, the term "stringent conditions" means the following conditions: (1) hybridization and washing under low ionic strength and high temperature, such as 0.2xSSC, 0.1% SDS, 60°C; (2) hybridization after adding denaturants, such as 50% (v/v) formamide, 0.1% bovine serum/0.1% Ficoll, 42°C; or (3) hybridization of two sequences sharing at least 95%, preferably 97% homology. Further, the hybridizing polynucleotides encode a polypeptide which retains the same biological function or activity as the mature polypeptide of SEQ ID NO:2

The invention also relates to nucleic acid fragments hybridized with the hereinabove sequence. As used herein, the length of "nucleic acid fragment " is at least 15bp, preferably 30bp, more preferably 50bp, and most preferably at least 100bp. These fragments can be used in the amplification techniques of nucleic acid, e.g., PCR, to determine and/or isolate the Rhor encoding polynucleotide.

The polypeptide and polynucleotide of the invention preferably are provided in isolated form, more preferably are purified to be homogeneous.

The full-length Rhor nucleotide sequence or its fragment can be prepared by PCR amplification, recombinant method and synthetic method. For PCR amplification, one can obtain said sequences by designing primers based on the nucleotide sequence disclosed herein, especially the ORF, and using cDNA library commercially available or prepared by routine techniques in the art as a template. When the sequence is long, it is usually necessary to perform two or more PCR amplifications and link the amplified fragments together correctly.

Once the sequence is obtained, one can produce lots of the sequences by recombinant methods. Usually, said sequence is cloned into a vector which is then transformed into a host cell. The sequence is isolated from the amplified host cells using conventional techniques.

Further, the sequence can be synthesized, especially when the fragments are short. Typically, several small fragments are synthesized and linked together to obtain a long sequence.

It is completely feasible to chemically synthesize the DNA sequence encoding the protein of invention, or the fragments or derivatives thereof. In addition, the mutation can be introduced into the protein sequence by chemical synthesis.

The amplification of DNA/RNA by PCR is preferably used to obtain Rhor gene. The primers used in PCR can be properly selected according to the sequence information disclosed herein and synthesized by the conventional methods. The amplified DNA/RNA fragments can be isolated and purified by conventional methods, e.g., gel electrophoresis.

The invention further relates to a vector comprising the polynucleotide of invention, a genetic engineered host cell transformed with the vector or the sequence encoding Rhor protein, and the

method for producing the Rhor polypeptide by recombinant techniques.

The recombinant Rhor polypeptides can be expressed or produced by the conventional recombinant DNA technology (Science, 1984; 224:1431), using the polynucleotide sequence of invention. Generally, it comprises the following steps:

(1) transfecting or transforming the appropriate host cells with the polynucleotide encoding Rhor polypeptide or the vector containing the polynucleotide;

(2) culturing the host cells in an appropriate medium;

(3) isolating or purifying the protein from the medium or cells.

In the present invention, the polynucleotide sequences encoding Rhor protein may be inserted into a recombinant expression vector. The term "expression vector" refers to a bacterial plasmid, bacteriophage, yeast plasmid, plant virus or mammalian cell virus, such as adenovirus, retrovirus or any other vehicle known in the art. Vectors suitable for use in the present invention include, but are not limited to, the T7-based expression vector for expression in bacteria (Rosenberg, et al., Gene, 56:125, 1987) , the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, J Biol. Chem., 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. On the whole, any plasmid or vector can be used to construct the recombinant expression vector as long as it can replicate and is stable in the host. One important feature of expression vector is that the expression vector typically contains an origin of replication, a promoter, a marker gene as well as the translation regulatory components.

The methods known by the artisans in the art can be used to construct an expression vector containing the DNA sequence of Rhor and appropriate transcription/translation regulatory components. These methods include *in vitro* recombinant DNA technique, DNA synthesis technique, *in vivo* recombinant technique and so on (Sambrook, et al. Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory. New York, 1989). The DNA sequence is efficiently linked to the proper promoter in an expression vector to direct the synthesis of mRNA. The exemplary promoters are lac or trp promoter of E. coli; P_L promoter of lamda-phage; eukaryotic promoter including CMV immediate early promoter, HSV thymidine kinase promoter, early and late SV40 promoter, LTRs of retrovirus and some other known promoters which control the gene expression in the prokaryotic cells, eukaryotic cells or virus. The expression vector may further comprise a ribosome-binding site for initiating the translation, transcription terminator and the like.

The expression vector preferably comprises one or more selective marker genes to provide a phenotype for selecting the transformed host cells, e.g., the dehydrofolate reductase, neomycin resistance gene and GFP (green flurencent protein) for eukaryotic cells, as well as tetracycline or ampicillin resistance gene for *E. coli*.

The vector containing said DNA sequence and proper promoter or regulatory elements can be transformed into appropriate host cells to express the protein.

The "host cell " includes prokaryote, e.g., bacteria; primary eukaryote, e.g., yeast; advanced

eukaryotic, e.g., mammalian cells. The representative examples are bacterial cells, e.g., *E. coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, e.g., yeast; plant cells; insect cells e.g., Drosophila S2 or Sf9; animal cells e.g., CHO, COS, 293 cells or Bowes melanoma, etc.

Transcription of the polynucleotide in higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10-300bps, that act on a promoter to increase gene transcription. Examples include SV40 enhancer on the late side of replication origin 100 to 270 bp, the polyoma enhancer on the late side of replication origin, and adenovirus enhancers.

The artisans know clearly how to select appropriate vectors, promoters, enhancers and host cells.

Recombinant transformation of host cell with the DNA might be carried out by conventional techniques known to the artisans. Where the host is prokaryotic, e.g., E. coli, the competent cells capable of DNA uptake, can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method using known procedures. Alternatively, $MgCl_2$ can be used. The transformation can also be carried out by electroporation. When the host is an eukaryote, transfection of DNA such as calcium phosphate co-precipitates, conventional mechanical procedures e.g., micro-injection, electroporation, or liposome-mediated transfection may be used.

The transformants are cultured conventionally to express Rhor polypeptide of invention. According to the used host cells, the medium for cultivation can be selected from various conventional mediums. The host cells are cultured under a condition suitable for its growth until the host cells grow to an appropriate cell density. Then, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

In the above methods, the recombinant polypeptide may be included in the cells, or expressed on the cell membrane, or secreted out. If desired, the physical, chemical and other properties can be utilized in various isolation methods to isolate and purify the recombinant protein. These methods are well-known to the artisans and include, but are not limited to conventional renaturation treatment, treatment by protein precipitant (e.g., salt precipitation), centrifugation, cell lysis by osmosis, sonication, supercentrifugation, molecular sieve chromatography or gel chromatography, adsorption chromatography, ion exchange chromatography, HPLC, and any other liquid chromatography, and the combination thereof.

Therefore, the recombinant human Rhor protein or polypeptide have various uses including, but not to be limited to: curing disorders caused by low or no activity of Rhor protein (e.g., baldness), and screening out antibodies, polypeptides or ligands as agonists or antagonists of Rhor. For example, antibodies can be used to activate or inhibit the function of Rhor protein. The expressed recombinant Rhor protein can be used to screen polypeptide library to find out therapeutically valuable polypeptide molecules which inhibit or activate Rhor protein.

In another aspect, the invention also includes polyclonal and monoclonal antibodies (mAbs),

preferably mAbs, which are specific for polypeptides encoded by Rhor DNA or fragments thereof. By "specificity", it means an antibody which binds to the Rhor gene products or a fragments thereof. Preferably, the antibody binds to the Rhor gene products or fragments thereof and does not substantially recognize nor bind to other antigenically unrelated molecules. Antibodies which bind to Rhor and block Rhor protein and those which do not affect the Rhor function are included in the invention.

The invention includes intact monoclonal or polyclonal antibodies, and immunologically-active antibody fragments, e.g., a Fab' or $(Fab)_2$ fragment, an antibody heavy chain, an antibody light chain,, or a chimeric antibody.

The antibodies in the present invention can be prepared by various techniques known in the art. E.g.,, purified Rhor gene products, or its antigenic fragments can be administrated to animals (e.g., rabbit, mice and rat) to produce polyclonal antibodies. Similarly, cells expressing Rhor or its antigenic fragments can be used to immunize animals to produce antibodies. The antibody of the invention can be monoclonal antibodies (mAbs). The mAbs can be prepared using hybridoma technique. Antibodies comprise those which block Rhor function and those which do not affect Rhor function. Antibodies can be produced by routine immunology techniques and using fragments or functional regions of Rhor gene product prepared by recombinant methods or synthesized by a polypeptide synthesizer. The antibodies binding to unmodified Rhor gene product can be produced by immunizing animals with gene products produced by prokaryotic cells (e.g., E. coli), and the antibodies binding to post-translationally modified forms thereof can be acquired by immunizing animals with gene products produced by eukaryotic cells (e.g., yeast or insect cells).

The antibody against Rhor can be used in immunohistochemical method to detect the presence of Rhor protein in biopsy specimen. The mAb can be radiolabelled and injected into body to trace the position and distribution of Rhor.

The polyclonal antibodies can be prepared by immunizing animals, such as rabbit, mouse, and rat, with Rhor protein. Various adjuvants, e.g., Freund's adjuvant, can be used to enhance the immunization.

The substances which act with Rhor protein, e.g., receptors, inhibitors, agonists and antagonists, can be screened out by various conventional techniques, using Rhor protein.

The Rhor protein, antibody, inhibitor, agonist or antagonist of the invention provide different effects when administrated in therapy. Usually, these substances are formulated with a non-toxic, inert and pharmaceutically acceptable aqueous carrier. The pH typically is about 5-8, preferably 6-8, although pH may alter according to the property of the formulated substances and the diseases to be treated. The formulated pharmaceutical composition is administrated in conventional routes including, but not limited to, oral, intramuscular, intraperitoneal, intravenous, subcutaneous, intradermal or topical administration.

The Rhor polypeptide can be directly used for curing disorders, e.g., baldness. The Rhor protein can be administrated in combination with other medicaments used for treating baldness.

The invention also provides a pharmaceutical composition comprising safe and effective amount of Rhor protein or its agonist or antigonist in combination with a pharmaceutically acceptable carrier. Such a carrier includes but is not limited to saline, buffer solution, glucose, water, glycerin, ethanol, or the combination thereof. The pharmaceutical formulation should be suitable for delivery method. The pharmaceutical composition may be in the form of injections which are made by conventional methods, using physiological saline or other aqueous solution containing glucose or auxiliary substances. The pharmaceutical compositions in the form of tablet or capsule may be prepared by routine methods. The pharmaceutical compositions, e.g., injections, solutions, tablets, and capsules, should be manufactured under sterile conditions. The active ingredient is administrated in therapeutically effective amount, e.g., about 1ug - 5mg/kg body weight per day. Moreover, the polypeptide of invention can be administrated together with other therapeutic agents.

When using pharmaceutical composition, the safe and effective amount of the Rhor protein or its antagonist or agonist is administrated to mammals. Typically, the safe and effective amount is at least about 10 ug/kg body weight and less than about 5 mg/kg body weight in most cases, and preferably about 10ug-1mg/kg body weight. Certainly, the precise amount depends upon various factors, such as delivery methods, the subject health, etc., and is within the judgment of the skilled clinician.

Rhor polynucleotides also have many therapeutic applications. Gene therapy technology can be used in the therapy of baldness, which is caused by the loss of Rhor expression or the expression of abnormal or non-active Rhor . The expression vectors derived from virus, such as retrovirus, adenovirus, adeno-associated virus, herpes simplex virus, parvovirus, and so on, can be used to introduce the Rhor gene into the cells. In addition, the recombinant Rhor gene can be packed into liposome and then transferred into the cells.

The methods for introducing the polynucleotides into tissues or cells include: directly injecting the polynucleotides into tissue in the body, *in vitro* introducing the polynucleotides into cells with vectors, such as virus, phage, or plasmid, and then transplanting the cells into the body.

The polypeptide molecule capable of binding Rhor protein can be obtained by screening out the random polypeptide library consisting of the various combinations of amino acids bound onto the solid matrix. In the screening procedure, Rhor protein should be labeled.

The invention further provides diagnostic assays for quantitative and *in situ* measurement of Rhor protein level. These assays are known in the art and include FISH assay and radioimmunoassay. The level of Rhor protein detected in the assay can be used to illustrate the importance of Rhor protein in diseases and to determine the Rhor-related diseases such as baldness.

A method of detecting Rhor protein in a sample by utilizing the antibody specifically against Rhor protein comprises the steps of: contacting the sample with the antibody specifically against Rhor protein; observing the formation of antibody complex which indicates the presence of Rhor protein in the sample.

The polynucleotide encoding Rhor protein can be used in the diagnosis and treatment of Rhor related diseases. In diagnosis, the polynucleotide encoding Rhor can be used to detect whether Rhor is expressed or not, and whether the expression is normal or abnormal in the case of diseases. Rhor DNA sequences can be used in the hybridization with biopsy samples to determine Rhor expression. The hybridization methods include Southern blotting, Northern blotting and *in situ* blotting, etc., which are public and sophisticated techniques. The corresponding kits are commercially available. A part of or all of the polynucleotides of the invention can be used as probe and fixed on a microarray or DNA chip for analyzing the differential expression of genes in tissues and for the diagnosis of genes. The Rhor specific primers can be used in RT-PCR and *in vitro* amplification to detect the transcripts of Rhor.

Detection of Rhor gene mutation is useful for the diagnosis of Rhor related diseases. The mutation forms of Rhor include site mutation, translocation, deletion, rearrangement and any other mutations compared with the wild-type Rhor DNA sequence. The conventional methods, e.g., Southern blotting, DNA sequencing, PCR and *in situ* blotting, can be used to detect mutation. Moreover, mutation sometimes affects protein expression. Therefore, Northern blotting and Western blotting can be used to indirectly determine the gene mutation. The experiments of the invention has indicated that the mutation of Rhor is directly related to baldness.

In one embodiment of the invention, a new gene was determined based on the phenotype and genetic analysis on the mice without fair, mice with little fair and mice with normal fair. The corresponding polynucleotide sequence was isolated. It encoded a polypeptide having the amino acid sequence shown in SEQ ID NO: 2. The polynucleotide of the invention was shown in SEQ ID NO: 1. The full-length sequence comprises 2484 bps, while the ORF is nucleotides 1-2481 and encodes a Rhor protein having 827amino acids (SEQ ID NO: 2). The studies showed that the mutation of the gene results in the blocking of signaling, thereby influencing the development of follicular cells and causing the congenital baldness in mice. Because of the high homology in the encoding region of Rhor protein, the partial deletion of the corresponding human gene in the genome is likely to cause the congenital baldness or little hair.

The Rhor provides new approach for curing diseases such as baldness, thus having huge potential applications.

The invention is further illustrated by the following examples. It is appreciated that these examples are only intended to illustrate the invention, but not to limit the scope of the invention. For the experimental methods in the following examples, they are performed under routine conditions, e.g., those described by Sambrook. et al., in Molecule Clone: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989, or as instructed by the manufacturers, unless otherwise specified.

Example 1

Mapping the baldness related gene

A hairless mutation was found in wildtype Balb/c, heterozygous was sparse hair, while homozygote was hairless. All the descendants of homozygote and wild type mice showed sparse hair phenotype. Inbred of heterozygous showed segregation of character and the ratio of phenotype representation Medel's reulavity of segregation, wild type : sparse hair: hairless was 1:2:1. Further, according to the pedigree analysis, this mutation was a single gene incomplete dominance.

Genomic DNA of 2000 mice was extracted for genotyping, and the data were analyzed by computer using the software Linkage. The mutation was mapped to a region between two microsatellite marker D11mit103 and D11mit338, and the mapping range was 1,700kb. The precision map was then generated to narrowing the mutation to a region of 800kb.Scaning the genome was carried out by sequencing the whole region, thereby mapping the mutation to a narrow region.

Example 2

Genomic DNA deletion assay

According to the standard method described in Molecular Cloning, the genomic DNA was extracted from hairless, sparse hair and wildtype mice tissue. The following primers were used.

Forward primer: 5' gcaggctagcgtgttaaagg 3'(SEQ ID NO:3)

Reverse primer: 5' aaaacggggtcatagcagc 3' (SEQ ID NO:4)

The mice DNA was used as templates to perform polymerase chain reaction with the Taq Gold polymerase commercial available from Applied Bio-system under the following thermal conditions:

Hot start of Taq polymerase and template denaturing: 95°C 10min

first round of thermal cycle:

95°C 30sec

68°C 45sec

perform 1°C decrease per cycle

72°C 2min30sec

repeat 12 times

second round of thermal cycle:

95°C 30sec

56°C 45sec

72°C 2min30sec

repeat 35 times

72°C 10 min

The PCR product was analyzed on a 1.0% agarose gel followed by ethidium bromide staining (Fig. 1). Product of heterozygous showed two bands, matching the bands from wildtype mice and homozygote hairless mice.

Example 3

Rhor gene genomic sequencing

In this Example, the genomic sequencing was performed to confirm the correlation between Rhor and hairless phenotype.

The PCR product in Example 2 was purified by Omega Cycle-Pure Kit. The following primers were used.

Forward primer: 5' gcacatctgagggaaggaag 3'(SEQ ID NO: 5)

Reverse primer: 5' cttccggtcaaatgcaaagt 3'(SEQ ID NO: 6)

The sequence was performed on the 3100 automatic sequencer, using BigDye terminal reagent purchased from Applied Biosystem and the following thermal conditions:

Template denature 98°C 2min

96°C 20sec

51°C 20sec

60°C 4min

repeat 25 times

Result of sequencing:

AGCCTACCTG AAGAGTGTCA GCCTACAGGA GCCCCGGGGA CGATGGCAGG AGGGCGCAGA 60
GAAGCGCCCC GGCTTCCGCC GCCAGGCCTC CCTGTCCCAG AGCATCCGCA AGAGCACAGC 120
CCAGTGGTTT GGGGTCAGCG GCGACTGGGA GGGCAAGCGA CAAAACTGGC ATCGTCGCAG 180
CCTGCACCAC TGCAGCGTGC ACTATGGCCG CCTCAAGGCC TCGTGCCAGA GAGAACTGGA 240
GCTGCCCAGC CAGGAGGTGC CATCCTTCCA GGGCACTGAG TCTCCAAAAC CGTGCAAGAT 300
GCCCAAGATT GTGGATCCAC TGGCTCGGGG TAGGGCCTTC CGCCATCCAG ATGAGGTGGA 360
CCGGCCTCAC GCTGCCCACC CACCTCTGAC TCCAGGGGTC CTGTCTCTCA CATCCTTCAC 420
CATGTCCGCT CTGGCTACTC CCATCTGCCC CGCCGCAAGA GGATATCTGT TGCCCATATG 480
AGCTTTCAGG CAGCCGCCGC CCTCCTCAAG GGGCGTTCCG TGCTAGATGC GACTGGGCAG 540
CGGTGCCGGC ATGTCAAACG CAGCTTCGCT TACCCCAGCT TCCTGGAGGA GGATGCTGTC 600
(SEQ ID NO: 7)

Comparing the sequence of wildtype mice with that of homozygote hairless mice, it could be seen that there was a 230bp deletion in the genome sequence of hairless mice (underlined portion).

Example 4

Obtain the complete ORF of Rhor

Using Total RNA extract kit from Qiagen, the mice skin total RNA was extracted and the RNA was analyzed on a 1% agarose gel and converted to cDNA by reverse transcription using Reverse Transcription System from Promega.

The following primers was used in PCR reaction:

Forward primer: 5' ACTCTGCTCTCAGCCGCTT 3' (SEQ ID NO: 8)

Reverse primer: 5' CCAGACACATGCTGGAGCTA 3' (SEQ ID NO: 9)

Polymerase: Takara LA polymerase

PCR thermal condition:

95 °C 30 sec

54 °C 45 sec

72 °C 3min
repeat 45 times
72°C 10min

The product was separated on a 0.8% agarose electrophoresis. The gel was cut and purified , thereby obtaining the target band from gel. The full length ORF of Rhor was obtained by sequencing.

The Rhor full length ORF was shown in SEQ ID NO:1 and the encoded amino acid sequence was shown in SEQ ID NO:2.

Example 5

Rhor cDNA deletion assay

The cDNA from Example 4 was used as template and the PCR reaction was performed using Taq Gold polymerase and the following primers:

Forward primer: 5' gcacatctgagggaaggaag 3'(SEQ ID NO: 10)
Reverse primer: 5' cttccggtcaaatgcaaagt 3' (SEQ ID NO: 11)
PCR thermal condition:
Hot start of Taq polymerase and template denaturing: 95°C 10min

First round of thermal cycle
95 °C 30sec
68 °C 45sec
perform 1°C decrease per cycle
72°C 90sec
repeat 14 times
second round of thermal cycle
95°C 30sec
54°C 45sec
72°C 90sec
repeat 40 times
72°C 10min

The products were analyzed on a 0.8% agarose gel and purified using Cycle-Pure Kit from Omega. The sequencing reaction was carried out using the same primer and BigDye terminal reagent from Applied Biosystem :

template denature 98°C 2min
96°C 20sec
51°C 20sec
60°C 4min
repeat 25 times
Results:

GTGTCAGCCT ACAGGAGCCC CGGGGACGAT GGCAGGAGGG CGCAGAGAAG CGCCCCGGCT 60
TCCGCCGCCA GGCCTCCCTG TCCCAGAGCA TCCGCAAGAG CACAGCCCAG TGGTTTGGGG 120

TCAGCGGCGA CTGGGAGGGC AAGCGACAAA ACTGGCATCG TCGCAGCCTG CACCACTGCA 180
GCGTGCACTA TGGCCGCCTC AAGGCCTCGT GCCAGAGAGA ACTGGAGCTG CCCAGCCAGG 240
AGGTGCCATC CTTCCAGGGC ACTGAGTCTC CAAAACCGTG CAAGATGCCC AAGATTGTGG 300
ATCCACTGGC TCGGGGTAGG GCCTTCCGCC ATCCAGATGA GGTGGACCGG CCTCACGCTG 360
CCCACCCACC TCTGACTCCA GGGGTCCTGT CTCTCACATC CTTCACCATG TCCGCTCTGG 420
CTACTCCCAT CTGCCCCGCC GCAAGAGGAT ATCTGTTGCC CATATGAGCT TTCAGGCAGC 480
CGCCGCCCTC CTCAAGGGGC GTTCCGTGCT AGATGCGACT GGGCAGCGGT GCCGGCATGT 540
CAAACGCAGC TTCGCTTACC CCAGCTTCCT GGAGGAGGAT GCTGTCGATG GAGCTGACAC 600
(SEQ ID NO: 12)

The underlined sequence showed the cDNA deletion in homozygote hairless mice, which was the same as that deletion in genomic sequence.

Example 6

Rhor coding protein structure analyze

Structure analysis showed the encoded protein of Rhor contained two highly conserved domains:

(1) The amino acids 619-759 in Rhor encoded protein formed a Rhomboid domain, suggesting that Rhor protein was a Rhomboid protein family member. The members of this family were found in bacteria and eukaryotes and most of them were membrane proteins. These proteins contained three highly conserved histidines in the putative transmembrane regions that might be involved in the peptidase function.

(2) The amino acids 610-804 in Rhor encoded protein formed a competence structure domain, suggesting Rhor protein was a member of the competence protein family. Most of the member in this family were membrane proteins. They had six helix trans-membrane structures. These protein transported nucleic acid through the membrane. Some of the family members were reported as critical elements when bacterium intakes extracellular DNA. All family members had an amino-terminal trans-membrane region, and two histidines in this region formed a highly conserved motif as the binding site for metal ion.

The above structural analysis showed that the mutated Rhor protein might be functionally defective in transporting message RNA, thereby blocking the hair-follicle development.

Example 7

Multiple functions of Rhor

Homozygote hairless mice were leaner than wild type mice. The axungia reservation was seldom found in dissection, suggesting that Rhor might also participate in axungia metabolism and the mutation would cause axungia metabolic disorder.

Further, the lymphadenoma was found during the hairless mice life span and lymphocyte lost its normal growing regulation. This suggested that Rhor could be in the regulation point of multiple pathways.

Example 8

Rhor expression in prokaryocyte

The following primers was synthesized:

forward primers 5'CGGATCCATGGCCTCAGCTGACAAGAATGGCAGCAACCTCCCA

3'(SEQ ID NO:13) (*Bam*HI recognition site GGATCC was introduced and the first C was protective base)

reverse primer: 5'ATAAAGCTTGCTCGATCTGGTCCACGATGTGATT 3'(SEQ ID NO: 14)(*Hind*III recognition site AAGCTT was introduced and ATA were protective bases)

The mice cDNA was used as template in the amplification of DNA encoding Rhor protein.

*Bam*HI and Hind III recognition sites were used to clone the PCR product into expression vector pET32a(Novagen) digested with the same enzymes. The vector was transformed into host *E.Coli:BL21(DE3)*. Transformed host expressed the foreign protein when induced by IPTG.

The expressed protein had a molecular weight of about 90Kda in SDS-PAGE.

Example 9

Antibody Preparation

The protein obtained in Example 8 was used as an antigen to prepare polyclonal antibody in rabbits. The procedure was as follows: The rabbits were immunized with 240 ug protein per rabbit in complete Freund's adjuvant. The rabbits were immunized again after four weeks with 140ug protein per rabbit in incomplete Freund's adjuvant. Finally, the rabbits were immunized two weeks later with 120ug protein per rabbit in incomplete Freund's adjuvant.

All the documents cited herein are incorporated into the invention as reference, as if each of them is individually incorporated. Further, it would be appreciated that, in the above teaching of the invention, the skilled in the art could make certain changes or modifications to the invention, and these equivalents would still be within the scope of the invention defined by the appended claims of the present application.